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The Effectiveness of Processed Grapefruit-Seed Extract as An Antibacterial Agent: II. Mechanism of Action and *In Vitro* Toxicity

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ABSTRACT

Objectives: Recent testimonials report grapefruit-seed extract, or GSE® (Citricidal®) to be effective against more than 800 bacterial and viral strains, 100 strains of fungus, and a large number of single and multicelled parasites. This study investigated GSE for antibacterial activity at varying time intervals and concentration levels and tissue toxicity at varying concentrations in an effort to determine if a concentration existed that was both microbicidal and nontoxic and in what period of time.

Design: Gram-negative and gram-positive isolates were introduced into graduated dilutions of GSE (twofold concentrations ranging from 1:1, through 1:512) for determination of bacterial activity. *In vitro* assays with human skin fibroblast cells were also performed at the same dilutions to determine toxicity.

Results: These tests indicated that from the 1:1 through the 1:128 concentrations, GSE remained toxic as well as bactericidal. However, test results indicated that at the 1:512 dilution, GSE remained bactericidal, but completely nontoxic.

Conclusions: The initial data shows GSE to have antimicrobial properties against a wide range of gram-negative and gram-positive organisms at dilutions found to be safe. With the aid of scanning transmission electron microscopy (STEM), the mechanism of GSE's antibacterial activity was revealed. It was evident that GSE disrupts the bacterial membrane and liberates the cytoplasmic contents within 15 minutes after contact even at more dilute concentrations.

INTRODUCTION

When bacterial species grow resistant to antibacterial agents, the significance of discovering substances that can provide continued antimicrobial effects is paramount (Lowbury, 1979). The problem with most antibacterial agents currently in use is that toxicity can overcome efficacy. These resistant bacteria are treated with antimicrobial agents that retard, diminish, or eliminate growth, but most cannot do so indefinitely without damaging tissue or retarding wound healing (Heggers et al., 1991, 1992). Scientists agree that an ideal an-

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Table 1. Bactericidal Index for Gram-Positive Organisms of Processed Grapefruit-Seed Extract

Organism	No.	Time (minutes)	Dilution[a]
Staphylococcus haemolytics	8	15	1:512
Staphylococcus aureus	4	15	1:256
Staphylococcus simulasis	1	15	1:32
Staphylococcus epidermidis	1	15	1:32
Enterococcus faecalis	2	30	1:32
Total	17		

[a]Dilution = lowest concentration of grapefruit-seed extract (GSE) that maintained its bacterial effect.

timicrobial substance must exhibit two crucial properties: be bactericidal and nontoxic. When a wound becomes colonized the danger of infection occurs. For patients susceptible to massive bacterial growth, such as those with thermal injuries, the threat of bacterial resistance is of critical concern (Lowbury, 1979). Many research studies have used a grapefruit-seed extract (GSE®; Citracidal,® BioChem Research, Lakeport, California) that has indicated that it is an outstanding substance whose therapeutic effect ranks equal to or better than known antibacterial agents. GSE is not only effective in treating infections, but also in decontaminating water, treating diarrhea, yeast infections, sore throats, and influenza. Also it has been shown effective in the disinfection of instruments and catheters in hospital settings (Kolmer *et al.*, 1951; United States. Navy Dept. Bureau of Medicine and Surgery, 1953). GSE reveals both traits of an ideal antimicrobial agent, safety and effectiveness. Inoescu et al. (1990) demonstrated that GSE performed as well as other antimicrobial agents tested on 770 strains of bacteria, and 93 strains of fungus. Our initial study of GSE provided scientific data that GSE exhibited superb antibacterial properties, when compared with other proven topical antibacterial agents (Reagor et. al, pp. 325–332, 2002). This study was designed to provide significant scientific evidence that GSE is not only safe to use but is an effective antimicrobial.

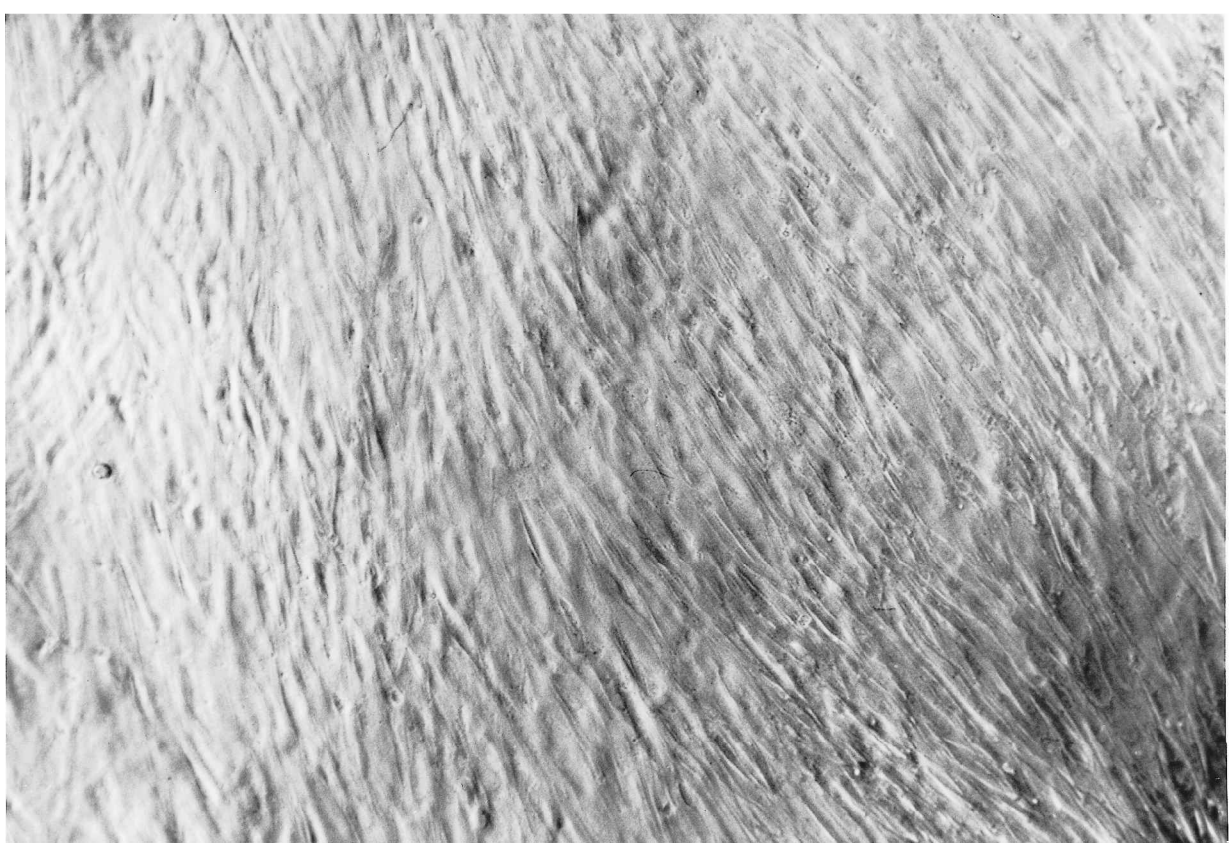

FIG. 1. *In vitro* (human fibroblast skin culture) normal untreated control.

Table 2. Bactericidal Index for Gram-Negative Organisms of Processed Grapefruit-Seed Extract

Organism	*No.*	*Time (minutes)*	*Dilution*[a]
Enterobacter cloacae	4	15	1:256
Escherichia coli	2	15	1:32
Acinetobacter baumanii/haemolyticus	8	15	1:256
Pseudomonas aeruginosa	5	15	1:512
Morganella morganii	1	15	1:32
Total	20		

[a]Dilution = lowest concentration of grapefruit-seed extract (GSE) that maintained its bacterial effect.

METHODS AND MATERIALS

Microorganisms

Ten (10) different species of gram-negative and gram-positive burn wound isolates from our patient population were used in our studies for a total of 37 different strains. Gram-positive organisms were maintained on sheep blood agar while the gram-negative organisms were maintained on MacConkey agar. Before the organisms were used in the *in vitro* study, the organisms were subcultured and incubated for 24 hours at 37°C. Each prepared suspension was compared to a turbidity standard (measured turbidities between the range 0.05–0.08) to ensure that an appropriate concentration of the organism was delivered to the test media (MicroScan,® 1991).

Grapefruit Seed Extract solutions

A solution of GSE was prepared by diluting twofold concentrations in Baxter Sterile Water for Irrigation, USP (Deerfield, Illinois). The concentrations (1:1, 1:2, 1:4, 1:8, 1:16 etc.) were prepared and diluted to 1:512 as the final dilution. Table 1 is representative of a certificate of analysis of Citricidal.

Grapefruit Seed Extract bacterial index

After time exposures of 15 minutes, 30 minutes, 60 minutes, 2 hours, and 4 hours, Sheep

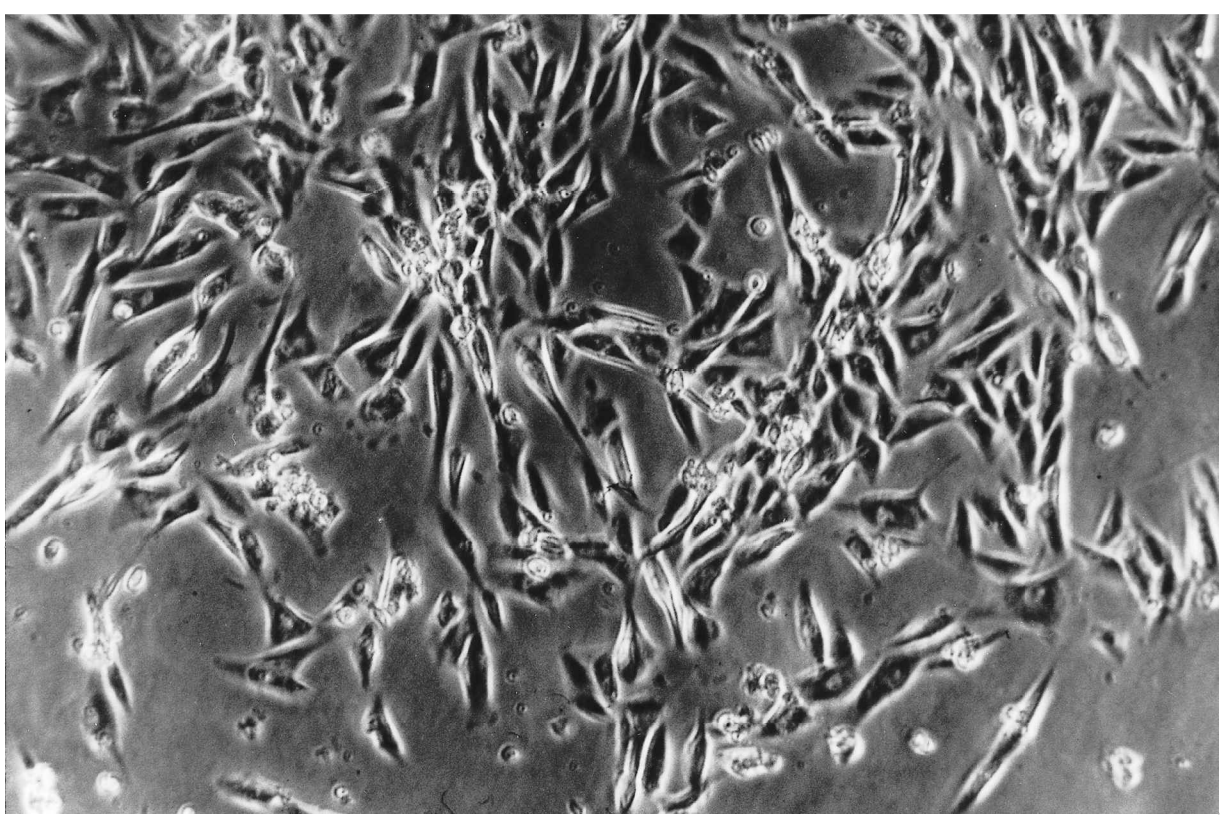

FIG. 2. *In vitro* 1:256 dilution of grapefruit-seed extract (GSE®)–treated human fibroblast.

Blood and MacConkey agar plates were inoculated with the test organisms from each dilution to determine the antimicrobial activity of GSE. All plates were incubated over night at 37°C. The bacterial index was determined to be that dilution that exhibited total inhibition of growth and represented the concentration of GSE that killed each organism within the time of exposure. This procedure is representative of the phenol coefficient (Kolmer et al, 1951; United States. Navy Dept. Bureau of Medicine and Surgery, 1953).

In vitro *toxicity assay*

Human skin fibroblast cells were cultured on minimal essential medium composed of 1000 μL and 10% serum. The cells were cultured and incubated in an atmosphere of 5% carbon dioxide in incubator (80% oxygen, 20% nitrogen) air at 37°C. When the cells reached confluence, they were challenged by 10 μL solutions of GSE at concentrations of 1:1, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256, and 1:512 and subsequently cultured for 3 days. After 3 days the cells were checked for viability and photomicrographs were taken. The fibroblast cells were then placed in fresh media absent of GSE and cultured for 5 days and subsequently checked for cell survival.

Electron microscopy

Electron microscopy (EM) examination compared bacteria treated with GSE versus nontreated bacteria (i.e., *Pseudomonas aeruginosa* and *Staphylococcus aureus*). For transmission electron microscopy (TEM), bacteria were fixed in 2% glutaraldehyde in 0.08 M of cacodylate buffer, pH 7.4. After overnight fixation, bacteria were washed and postfixed in 1% osmium tetroxide in 0.08 M of cacodylate. Samples were then dehydrated through ethanol and embedded in Spurr epoxy resin. After tissue embedding, 80-nm thick sections were obtained and stained with uranyl acetate and lead citrate. Sections were then observed with a Japanese Electron Optics, Ltd., 100CX TEM (Boston, MA) and pho-

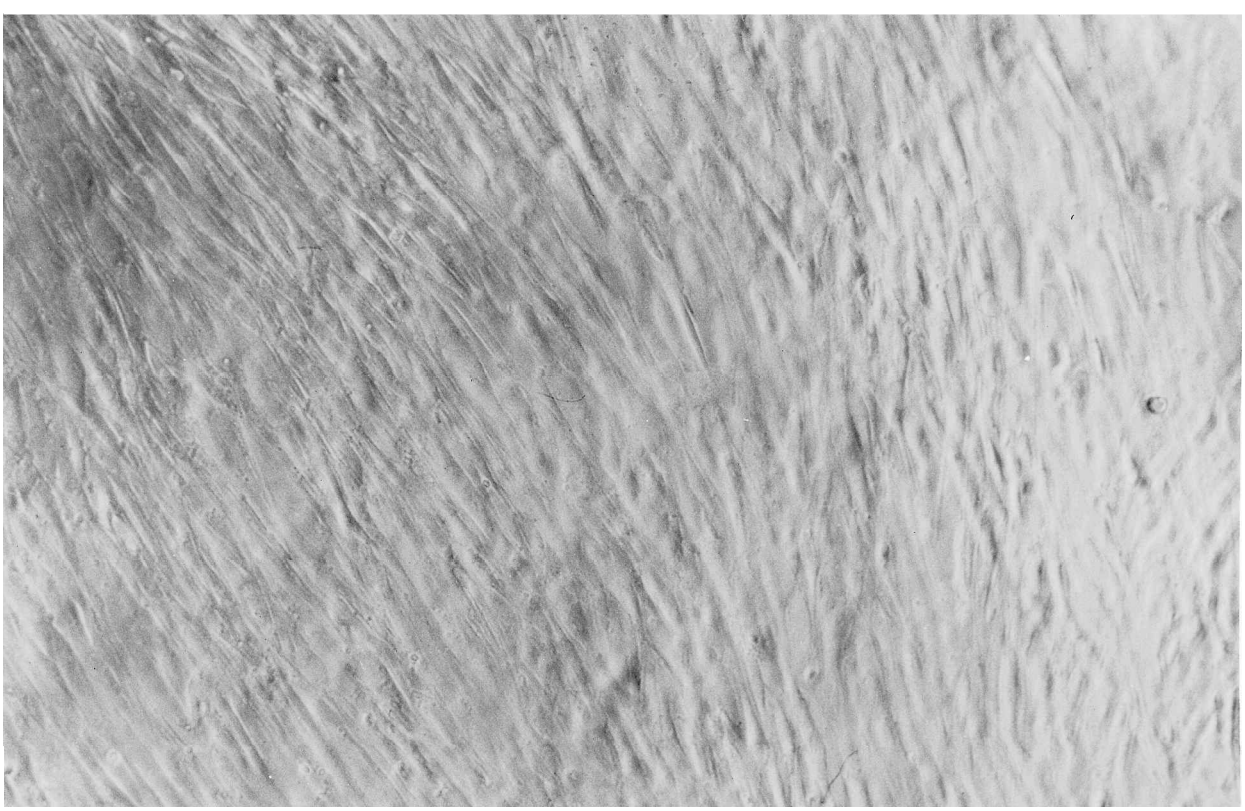

FIG. 3. *In vitro* 1:512 dilution of grapefruit-seed extract (GSE®)–treated human fibroblast (note that viable cells are comparable to Figure 1).

tographed at 10,000×. The examination and photography of the bacteria were performed by a microscopist blinded to the treatment groups.

RESULTS/DISCUSSION

The data resulting from these experiments indicated that GSE exhibited a strong bactericidal effect at concentrations as dilute as 1:512 within a period of 15 minutes of contact (Tables 1 and 2). This activity was consistent for all gram-positive and gram-negative bacteria tested. Furthermore, the results obtained in these experiments support the data of the previous *in vitro* study of GSE (Reagor *et. al.*, 2002). As shown in the bactericidal index of gram-negative organisms (Table 2), *Enterobacter cloacae* assays 1,4, biotype 771017-2 and the *Acinetobacter baumannii* assays 2, 6, 7, biotypes 0006673-0 and 0206073-0, some plates demonstrated possible contamination based on the growth patterns presented when compared to the results of similar assays. *S. haemolyticus* exhibited susceptibility to all concentrations of GSE (up to the 1:512 dilution) with few exceptions, which again, may have been caused by contamination.

Human fibroblast skin cells were used to determine the concentration of GSE that would be nontoxic while remaining lethal to bacteria. Concentrations from 1:1 through 1:256 proved to be highly to partially toxic to the fibroblasts, albeit bactericidal. The fibroblast cells that were treated with the concentrations from 1:256 through 1:512 remained viable (Figs. 1–4). EM was used to elucidate the mechanism in which GSE destroys the bacteria. The examination compared bacteria treated with an initial concentration of GSE (1:512) versus nontreated bacteria (i.e., *P. aeruginosa* and *S. aureus*). These studies disclosed that all concentrations of GSE caused cell death by disruption of the cell membrane of the bacteria (Fig. 5B and 6B). Dr. Sung-Hwan stated that, "Considering all the elec-

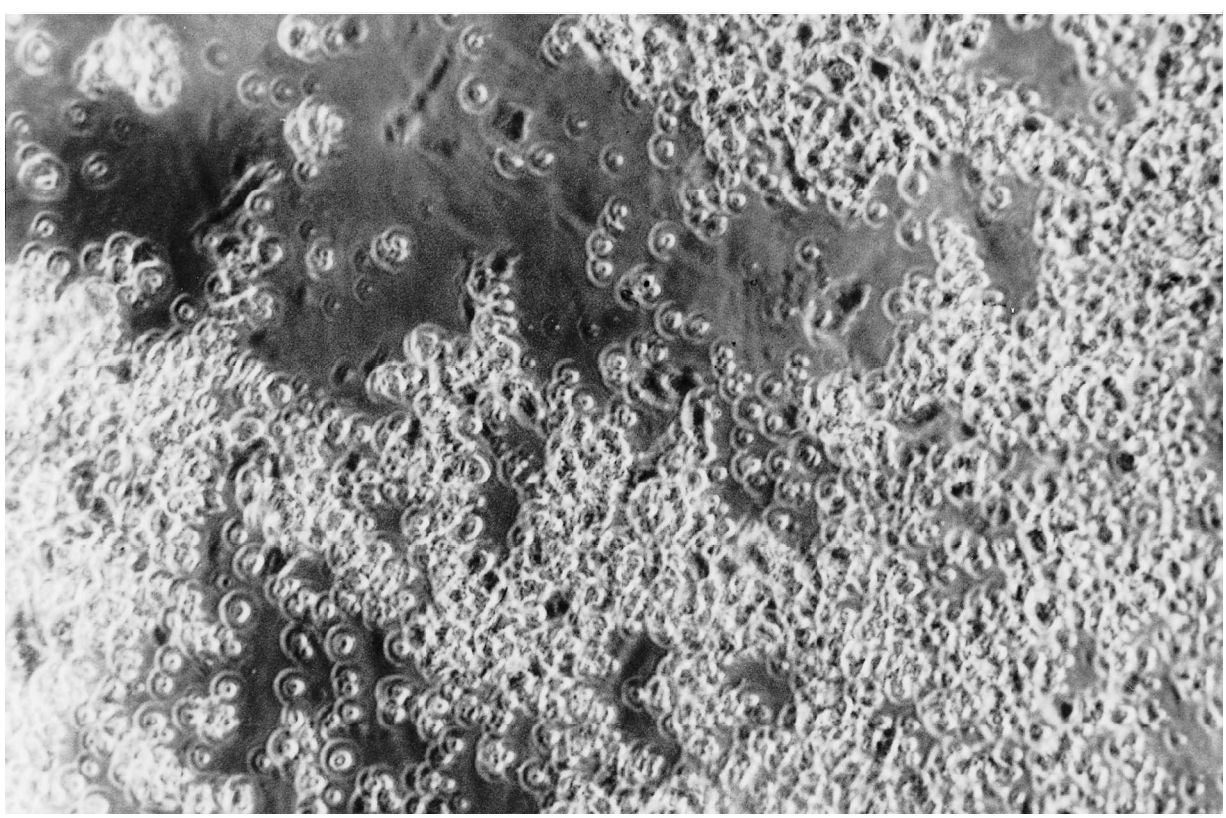

FIG. 4. *In vitro* 1:1 dilution of grapefruit-seed extract (GSE®)–treated human fibroblast (note that no viable cells are present).

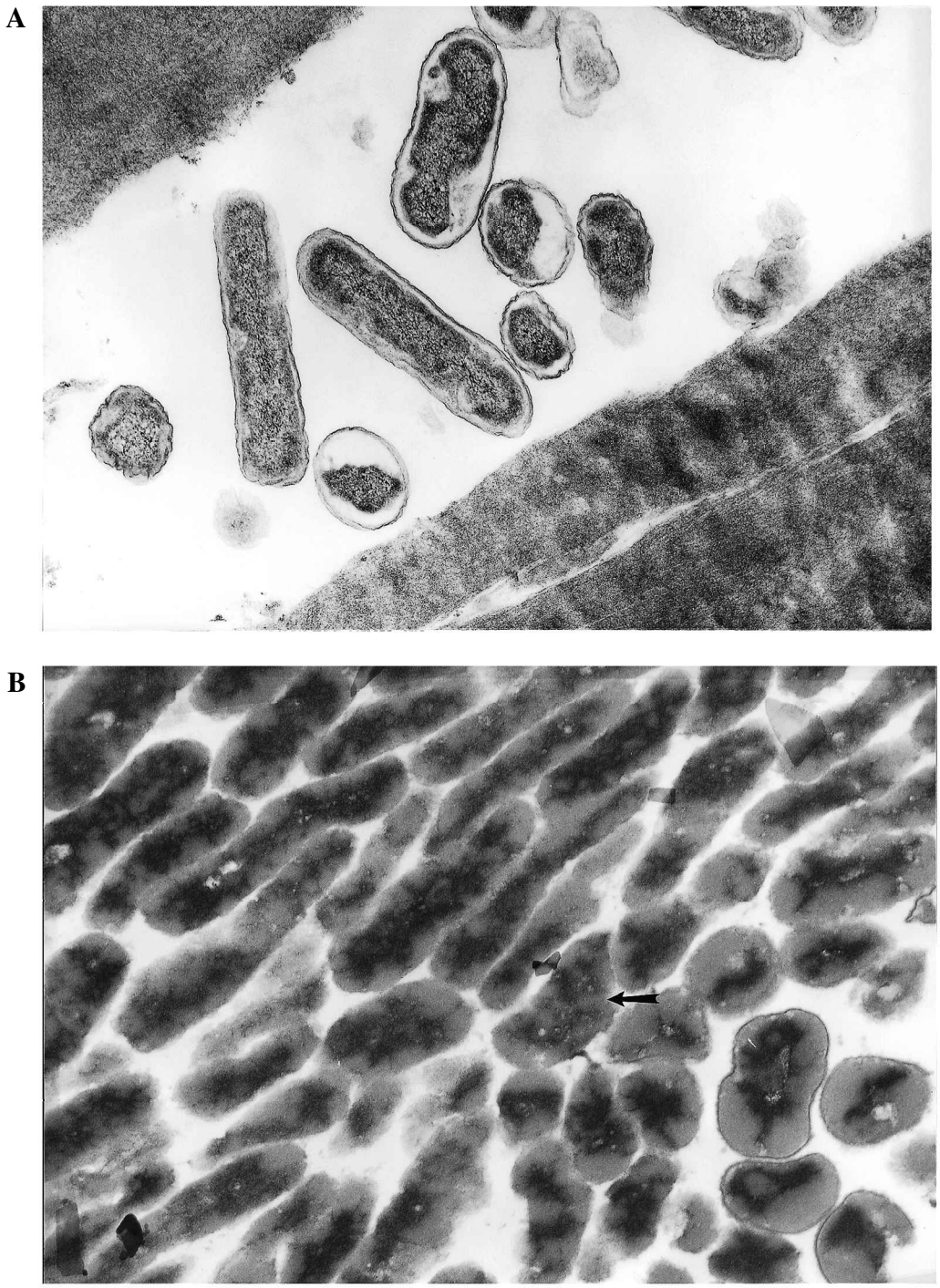

FIG. 5. **A.** Electron microscopy. Live *Pseudomonas aeruginosa* untreated (magnification 7,500×). **B.** Electron microscopy. Bactericidal effects of grapefruit-seed extract (GSE®) (*Pseudomonas aeruginosa*). Note disruption of membrane (arrow) (Magnification 7,500×).

tron micrographs, we believe that the microbial uptake of GSE alters the cell membrane by inhibiting enzymatic activities. You can see the loss of the cytoplasmic membrane" (Sung-Hwan Cho, personal communication). Human cells are eukaryotic, a cell with a defined nucleus surrounded by a cell membrane. Consequently this study supports the concept that application of GSE solutions undiluted would be detrimental to human eu-

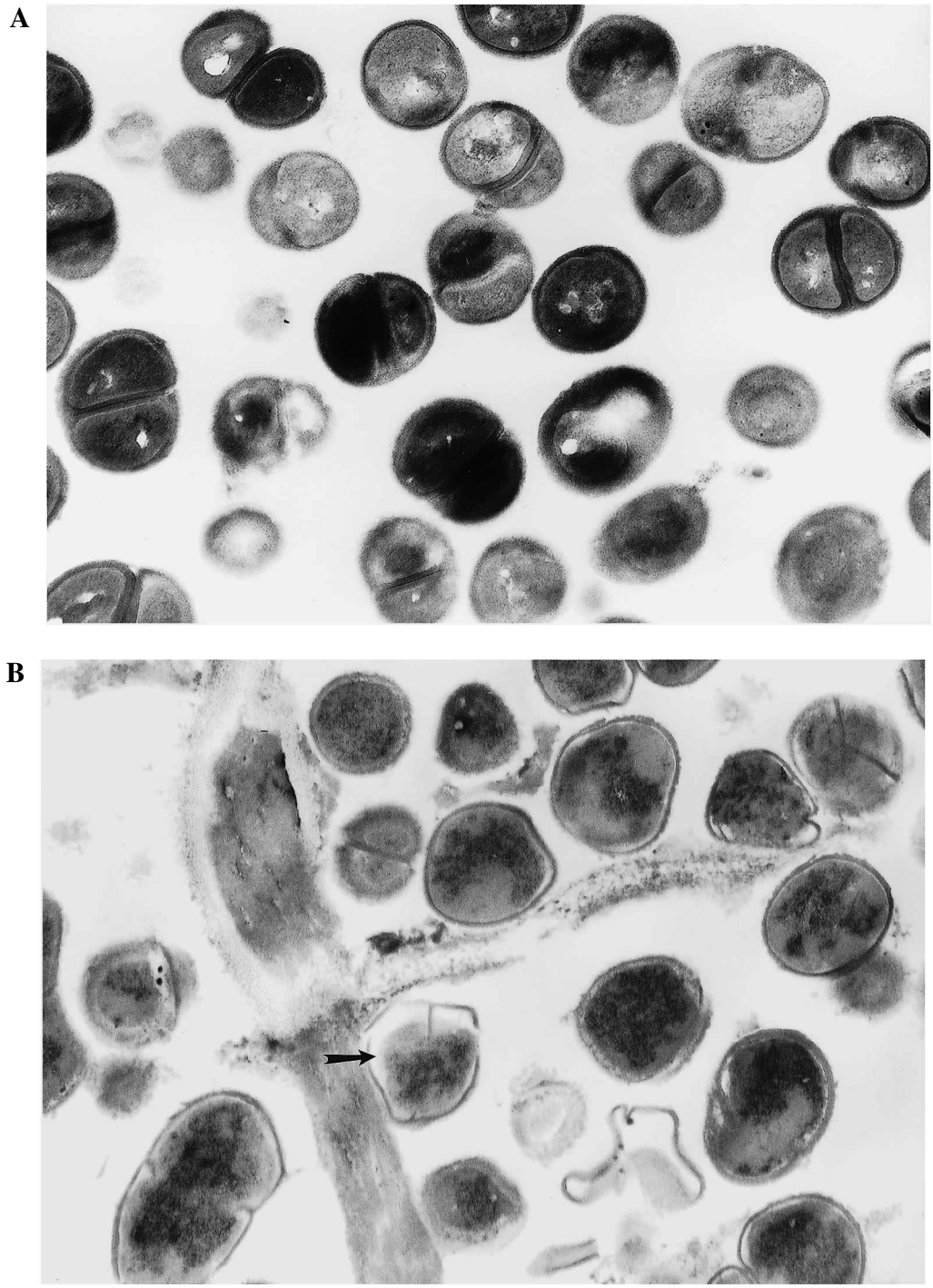

FIG. 6. **A.** Electron microscopy. Live *Staphylococcus epidermidis* untreated (magnification 7,500×). **B.** Electron microscopy. Bactericidal effects of grapefruit-seed extract (GSE®) (*Staphyloccus epidermidis*) (magnification 7,500×).

karyotic tissue. An *in vivo* animal infection study is anticipated in which GSE will be applied as a topical antibacterial agent to full thickness dermal burns infected with *P. aeruginosa* and *S. aureus.*

CONCLUSIONS

Our results indicate that GSE is lethal to gram-positive and gram-negative organisms alike, and substantiates a previous study (Reagor et.

al., 2002). Quite notable was the finding that GSE demonstrated antibacterial effects at concentrations as dilute as 1:512, which was also found to be nontoxic to human skin fibroblasts. The rapid lethality (15 minutes) of GSE for these organisms clearly indicated that the mechanism of action was membrane disruption as demonstrated by EM (Figs. 5B and 6B).

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